

IDENTIFICATION OF ESTER METABOLITES FROM PETROLEUM HYDROCARBON BIODEGRADATION IN GROUNDWATER USING GC×GC-TOFMS

KIRK T. O'REILLY,^{*,†} RACHEL E. MOHLER,[‡] DAWN A. ZEMO,[§] SUNGWOO AHN,[†] ASHEESH K. TIWARY,^{||}
RENAE I. MAGAW,^{||} CATALINA ESPINO DEVINE,^{||} and KAREN A. SYNOWIEC^{||}

[†]Exponent, Bellevue, Washington, USA

[‡]Chevron Energy Technology, Richmond, California, USA

[§]Zemo and Associates, Incline Village, Nevada, USA

^{||}Chevron Energy Technology, San Ramon, California, USA

(Submitted 3 February 2015; Returned for Revision 1 April 2015; Accepted 9 April 2015)

Abstract: In an effort to understand the nature and toxicity of petroleum hydrocarbon degradation metabolites, 2-dimensional gas chromatography linked to a time-of-flight mass spectrometer (GC×GC-TOFMS) was used to conduct nontargeted analysis of the extracts of 61 groundwater samples collected from 10 fuel release sites. An unexpected result was the tentative identification of 197 unique esters. Although esters are known to be part of specific hydrocarbon degradative pathways, they are not commonly considered or evaluated in field studies of petroleum biodegradation. In addition to describing the compounds identified, the present study discusses the role for nontargeted analysis in environmental studies. Overall, the low toxicological profile of the identified esters, along with the limited potential for exposure, renders them unlikely to pose any significant health risk. *Environ Toxicol Chem* 2015;34:1959–1961. © 2015 The Authors. Published by Wiley Periodicals, Inc., on behalf of SETAC.

Keywords: Ester Total petroleum hydrocarbons Petroleum metabolite Polar compound Groundwater

INTRODUCTION

Although esters are known to be potential metabolites of alkane biodegradation [1], they are not commonly evaluated when petroleum degradation in the field is assessed [2,3]. In an effort to better understand the nature and toxicity of polar compounds that are quantified and reported as petroleum hydrocarbons under some regulatory-required analytical methods, a 2-dimensional gas chromatography (GC×GC) linked to a time-of-flight mass spectrometer (TOFMS) was used to conduct nontargeted analysis of the extracts of groundwater collected from fuel release sites [4,5]. An unexpected result was the large number of esters tentatively identified in these samples. The purpose of the present short communication is to describe these findings and discuss their implications.

At some fuel release sites, there is a regulatory requirement to monitor the concentration of hydrocarbons dissolved in groundwater using analytical methods commonly referred to as total petroleum hydrocarbons as diesel, total extractable hydrocarbons, or diesel-range organics. These analyses typically are based on US Environmental Protection Agency (USEPA) method 3510C [6] for solvent extraction and USEPA method 8015B/C [7,8] for quantitation using gas chromatography with a flame ionization detector. The methods measure all extractable organics in a sample and are not specific to hydrocarbon compounds that originate from a fuel release; consequently they may overestimate the concentration of dissolved hydrocarbons in groundwater. Silica gel cleanup (using USEPA method

3630C [9] or a similar method) of the solvent extract prior to injection into the gas chromatography is recommended to separate polar compounds from the nonpolar petroleum hydrocarbons, thereby providing a more accurate measure of the dissolved hydrocarbons present. The metabolites of petroleum biodegradation are often the primary type of polar compound present in groundwater associated with a fuel release, and may make up the vast majority of the organics that are measured as total petroleum hydrocarbons as diesel without silica gel cleanup [10]. Some regulators limit the application of silica gel cleanup because of a lingering uncertainty about the characteristics and toxicity of the metabolites. Therefore, research efforts are ongoing to identify, quantify, and evaluate the potential toxicity of these metabolites in groundwater [4,5].

Aerobic metabolism of hydrocarbons is typically initiated with addition of oxygen to generate an alcohol through the action of an oxygenase enzyme. Depending on the position of the hydroxyl group, the alcohol is transformed into either an aldehyde or a ketone. Aldehydes are converted to acids through addition of water across the carbon–oxygen double bond. Ketones can be oxidized to acetyl esters through the action of another oxygenase enzyme [11]. Hydrolysis of the ester results in an acid and an alcohol. An important anaerobic degradation process is initiated by the addition of fumarate to generate an organic diacid that can undergo subsequent transformation [12]. A second anaerobic pathway is initiated by the subterminal carboxylation at the C-3 position of an alkyl chain, generating a monoacid [13]. Esters are not part of either anaerobic pathway.

Because organic acids are generated under both aerobic and anaerobic conditions, they are common targets of metabolite monitoring in the field [2,3,14]. Esters typically are not included as targeted analytes. Also, standard methods for organic acid analysis may destroy esters that may be present. Esters can be hydrolyzed when groundwater samples are acidified, either as a

All Supplemental Data may be found in the online version of this article.

This is an open access article under the terms of the Creative Commons Attribution License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited.

* Address correspondence to koreilly@exponent.com.

Published online 18 April 2015 in Wiley Online Library (wileyonlinelibrary.com).

DOI: 10.1002/etc.3022

preservative or as a method to increase the extraction efficiency of acids. Because of poor separation of organic acids using standard gas chromatography, they are typically derivatized using chemical processes that would hydrolyze esters to the corresponding acid and alcohol [2].

MATERIALS AND METHODS

Sixty-one groundwater samples were collected at 5 fuel terminals and 5 service-station sites affected by releases of diesel, or a mixture of gasoline and diesel (for the terminals), or gasoline (for the service stations). All the sites were located in California. Groundwater was collected from monitoring wells in both source and downgradient areas at each site. Samples were shipped on ice, but not otherwise preserved. Field blanks were not collected for the present study. Initial results from the terminals are described in more detail in Zemo et al. [5], and the results for esters and acids were combined in that study. Results from the subsequent larger data set, including the service-station sites, are presented for the first time in the present study. Natural attenuation parameters indicated anaerobic biodegradation conditions ranging from nitrate-reducing to methanogenic [5]. Each groundwater sample was extracted in triplicate using methylene chloride (USEPA method 3510 [6]). The pH of the groundwater at the time of collection was typically between 6.5 and 7.5, and was not adjusted prior to extraction. One methylene chloride extract for each sample was subjected to silica gel cleanup (USEPA method 3630 [9]) to separate the hydrocarbons from the polar compounds. The polar compounds that were retained on the silica gel column were eluted with 45 mL of methanol, and the methanol eluate was concentrated to 1 mL. The 3 methylene chloride extracts (without and with silica gel cleanup) and the methanol eluate from each well were analyzed on a Leco Pegasus IV GC×GC-TOFMS. Details on the columns used and temperature profiles are presented in Mohler et al. [4]. Leco ChromaTOF software was used to process the data and generate peak tables. Mass spectra generated by the GC×GC-TOFMS were compared with mass spectra in the National Institute for Standards and Technology library. Spectra that matched with a mass spectral similarity value greater than 750 were assigned tentative compound names and used to determine compound class.

RESULTS AND DISCUSSION

Approximately 1775 unique nonhydrocarbons were tentatively identified by GC×GC-TOFMS. Of these, 1403 (80%) unique compounds consisted only of oxygen, carbon, and hydrogen; the remaining also contained a sulfur and/or nitrogen atom. The 197 (11%) unique tentatively identified esters are listed in the Supplemental Data, and 18 example compounds are shown in Figure 1. Ester formation is not thought to be an artifact of the sample preparation process because extraction occurs at or near neutral pH. Additional details about other tentatively identified compounds are presented in Mohler et al. [4] and Zemo et al. [5]. For comparison, 113 (6%) unique compounds were tentatively identified as organic acids. The esters were subdivided further into the classes shown in Table 1. These compounds range in size from 2 to 35 carbons, with all but 1 being 24 carbons or fewer. Quantifying the environmental concentrations of most of these compounds is not currently possible, because standards are not available; therefore, extraction efficiency, percentage recovery, and analytical response could not be determined. Evaluation of 28 potential petroleum metabolites for which standards are available found

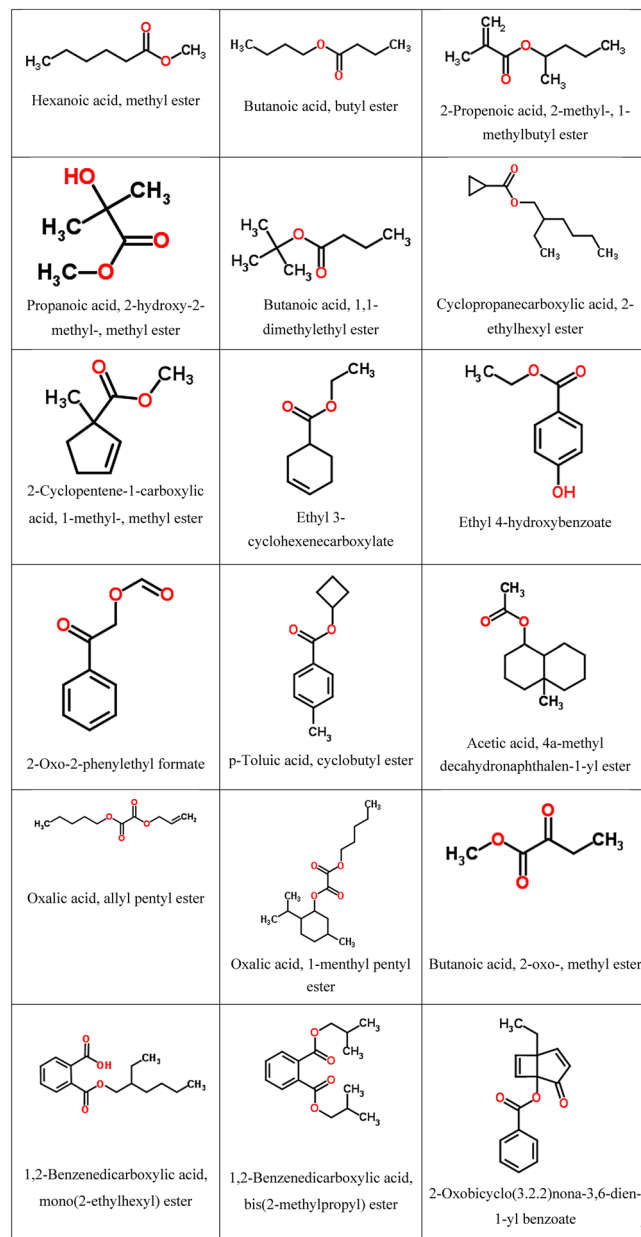


Figure 1. Eighteen examples of tentatively identified esters found in groundwater at fuel release sites. Structures obtained from ChemSpider [22]. See Supplemental Data for details.

their limit of quantitation to be in the range of 2 ng to 20 ng per injected volume [4].

The carbon structures of the esters, acid, and alcohol components include both small ($\leq C_4$) compounds consistent with traditional metabolism and those with more complex structures that are more apparent oxidation products of hydrocarbon degradation. These include compounds with alkyl, cycloalkyl, and aromatic structures. Of all the compounds, 42

Table 1. Subclasses of esters

Class	No. of unique compounds
Alkane esters	123
Aromatic esters	35
Cyclic esters	31
Polycyclic esters	5
Polyaromatic esters	3

were esters or diesters of the 2-carbon oxalic acid (see row 5 in Figure 1). Although 17 additional diacid esters were tentatively identified, they did not include any substituted succinic or fumaric acids that are commonly evaluated as indicators of anaerobic degradation [3,15].

The number of esters identified was unexpected, because esters are not commonly considered to be part of aerobic or anaerobic degradative pathways. With the limited information available, it is difficult to speculate on whether esters are generated for a specific purpose, such as carbon storage under electron acceptor limitation, use as surfactants to increase hydrocarbon availability, or as a means of eliminating structures that specific microbial strains cannot degrade. Comparisons of source area and downgradient wells indicate that the esters are transient and degrade within the plume [4,5].

The results of the present study indicate the value of conducting nontargeted analysis as part of some environmental investigations [4,16]. Although traditional targeted analysis allows for tracking and quantification of specific compounds, it may tell only a partial story. Advances in analytical technology, such as GC×GC and TOFMS, are allowing more detailed studies of the fate and toxicity of hydrocarbons in the environment [5,17]. Although the present study is focused on the metabolites of petroleum fuels in groundwater, similar tools are being used to study crude oil releases [18].

A key purpose of identifying the compounds in the present study was to evaluate the potential toxicity of the metabolites generated from petroleum degradation. Although toxicity data for esters is limited, a USEPA Office of Prevention, Pesticides, and Toxic Substances registration decision found no unreasonable adverse effects resulting from exposure to the aliphatic esters evaluated [19]. Because of the in vivo hydrolysis of esters to acids and alcohols, the High Production Volume Challenge Program of the USEPA [20] assigns an ester's toxicity based on its corresponding acid and alcohol using the read-across hazard characterization approach [21]. By applying a similar approach, the esters identified in groundwater collected at our sites were determined to have low potential for human toxicity [5]. Bioassay-based studies to evaluate the aquatic toxicity of groundwater containing petroleum metabolites, including esters, are ongoing.

SUPPLEMENTAL DATA

Tables S1 and S2. (238 KB PDF).

Acknowledgment—Financial support for the present study was provided by Chevron Environmental Management Company via the Remediation Technology Development Initiative.

Data availability—Data, associated metadata, and calculation tools are available in the Supplemental Data.

REFERENCES

1. Forney FW, Markovetz AJ. 1970. Subterminal oxidation of aliphatic hydrocarbons. *J Bacteriol* 102:281–282.
2. Barcelona MJ, Lu J, Tomczak DM. 1995. Organic acid derivatization techniques applied to petroleum hydrocarbon transformation in subsurface environments. *Ground Water Monit R* 15:114–124.
3. Young LY, Phelps CD. 2005. Metabolic biomarkers for monitoring in situ anaerobic hydrocarbon degradation environmental. *Environ Health Persp* 113:62–67.
4. Mohler RE, O'Reilly KT, Zemo DA, Tiwary AK, Magaw RI, Synowiec KA. 2013. Non-targeted analysis of petroleum metabolites in groundwater using GC×GC-TOFMS. *Environ Sci Technol* 47:10471–10476.
5. Zemo DA, O'Reilly KT, Mohler RE, Tiwary AK, Magaw RI, Synowiec KA. 2013. Nature and estimated human toxicity of polar metabolite mixtures in groundwater quantified as TPHd/DRO at biodegrading fuel release sites. *Ground Water Monit R* 33:44–56.
6. US Environmental Protection Agency. 1996. Method 3510C: Separatory funnel liquid-liquid extraction. Washington, DC. [cited 8 April 2015]. Available from: <http://www.epa.gov/epawaste/hazard/testmethods/sw846/pdfs/3510c.pdf>
7. US Environmental Protection Agency. 1996. Method 8015B: Nonhalogenated organics using GC/FID. [cited 8 April 2015]. Available from: <http://www.epa.gov/epawaste/hazard/testmethods/sw846/pdfs/8021b.pdf>
8. US Environmental Protection Agency. 2007. Method 8015C: Nonhalogenated organics by gas chromatography. [cited 8 April 2015]. Available from: <http://www.epa.gov/solidwaste/hazard/testmethods/sw846/pdfs/8015c.pdf>
9. US Environmental Protection Agency. 1996. Method 3630C: Silica gel cleanup. Washington, DC. [cited 8 April 2015]. Available from: <http://www.epa.gov/epawaste/hazard/testmethods/sw846/pdfs/3630c.pdf>
10. Zemo DA, Foote GR. 2003. The technical case for eliminating the use of the TPH analysis in assessing and regulating dissolved petroleum hydrocarbons in ground water. *Ground Water Monit R* 23:95–104.
11. Fritsche W, Hofrichter M. 2000. Aerobic degradation by microorganisms. In Klein J, ed, *Environmental Processes—Soil Decontamination*. Wiley-VCH, Weinheim, Germany, pp 146–155.
12. Grossi V, Cravo-Laureau C, Guyoneaud R, Ranchou-Peyruse A, Hirschler-Rea A. 2008. Metabolism of n-alkanes and n-alkenes by anaerobic bacteria: A summary. *Org Geochem* 39:1197–1203.
13. So CM, Phelps CD, Young LY. 2003. Anaerobic transformation of alkanes to fatty acids by a sulfate-reducing bacterium, Strain Hxd3. *Appl Environ Microbiol* 69:3892–3900.
14. Van Stempvoort DR, Millar K, Lawrence J. 2009. Accumulation of short-chain fatty acids in an aquitard linked to anaerobic biodegradation of petroleum hydrocarbons. *Appl Geochem* 24:77–85.
15. Beller HR, Ding WH, Reinhard M. 1995. Byproducts of anaerobic alkylbenzene metabolism useful as indicators of in situ bioremediation. *Environ Sci Technol* 29:2864–2870.
16. Hoh E, Dodder NG, Lehotay SJ, Pangallo KC, Reddy CM, Maruya KA. 2012. Nontargeted comprehensive two-dimensional gas chromatography/time-of-flight mass spectrometry method and software for inventorying persistent and bioaccumulative contaminants in marine environments. *Environ Sci Technol* 15:8001–8008.
17. Mao D, Lookman R, Van De Weghe H, Weltens R, Vanermen G, De Brucker N, Diels L. 2009. Combining HPLC-GC×GC, GC×GC/ToF-MS, and selected ecotoxicity assays for detailed monitoring of petroleum hydrocarbon degradation in soil and leaching water. *Environ Sci Technol* 43:7651–7657.
18. McKenna AM, Nelson RK, Reddy CM, Savory JJ, Kaiser NK, Fitzsimmons JE, Marshall AG, Rodgers RP. 2013. Expansion of the analytical window for oil spill characterization by ultrahigh resolution mass spectrometry: Beyond gas chromatography. *Environ Sci Technol* 47:7530–7539.
19. US Environmental Protection Agency. 2009. Screening-level hazard characterization: Terpenoid primary alcohols and related esters category. [cited 2014 October 2]. Available from: http://www.epa.gov/oppsrrd1/REDs/aliphatic_esters.pdf
20. US Environmental Protection Agency. 2007. Reregistration eligibility decision, aliphatic esters. EPA 738/R-07/002. Washington, DC.
21. Patlewicz G, Roberts DW, Aptula A, Blackburn K, Hubesch B. 2013. Workshop: Use of “read-across” for chemical safety assessment under REACH. *Regul Toxicol Pharmacol* 65:226–228.
22. Royal Society of Chemistry. 2015. ChemSpider. [cited 8 April 2015]. Available from: <http://www.chemspider.com/>